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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Doherty et al.
Filing Date: 20 January 1999
Serial No.: 09/234,208
For: HER-2 BINDING ANTAGONISTS
Art Unit: 1642
Examiner: Jennifer Hunt
Docket: 49321-1
Date: 22 November 2002

Assistant Commissioner for Patents
Washington, DC 20231

CONSIDERED
MLH
3/9/03

AFFIDAVIT OF DR. GAIL M. CLINTON UNDER 37 C.F.R. § 1.132
(IN SUPPORT OF AMENDMENT B UNDER37 CFR § 1.111)

Sir or Madam:

I, Dr. Gail Clinton, being duly sworn, say:

1. I am an original and true inventor of the subject matter described in the above-identified pending patent application.

2. I am an internationally recognized scientist and am presently employed as an Associate Professor at Oregon Health and Sciences University in Portland, Oregon (from 1/01/87 to present). I received a Bachelor of Science Degree in 1969 from the University of California, San Diego, and a Ph.D. degree from the University of California, San Diego in 1974. I completed a postdoctoral fellowship at Harvard Medical School in 1981.

3. I am an author or co-author of more than 45 peer-reviewed research articles and I am a member of a number of scientific and medical societies, most notably American Association of Cancer Research. I have received a number of prizes and awards for achievement in research, most notably I was the recipient of a distinguished postdoctoral fellowship from the

American Cancer Society, numerous grants from the National Cancer Institute, and was awarded a Fogarty Senior International Fellowship. I have served on several peer review groups and study sections and have been invited to give numerous presentations on my research at national and international meetings.

4. I have read the above-identified patent application, and understand that particular claims have been rejected under 35 U.S.C. § 112, ¶ 1, based on an alleged lack of enablement for *in vivo* utility. In particular, it appears that the Office does not fully appreciate the therapeutic significance, including the *in vivo* therapeutic significance, of the anchorage-independent (in soft agar) cancer cell growth experiments described at page 13 of the originally filed patent application involving SKOV-3 and 17-3-1 carcinoma cells. While the disclosed experiments of the application are *in vitro* cell culture experiments, the soft agar assay utilized is a widely recognized model system for human cancer (DiFore et al., *Science* 237:178-182, 1987; Hudziak et al., *Proc. Natl. Acad. Sci. USA* 84:7159-7163, 1987; and Baasner et al., *Oncogene* 13:901-911, 1996; all cited in said patent application, and all of record in this prosecution file as **EXHIBIT A** of the Affidavit of Dr. Edward Neuwelt which is being contemporaneously filed with the instant affidavit), and thus inhibition of such anchorage-independent growth in this system should be, and is within the relevant art taken as substantial proof of not only a well-established *in vivo* utility, but also a specific, credible and substantial *in vivo* utility. This is especially true where the therapeutic *target* HER-2 receptor is already a ***bona fide* clinical target** of the Herceptin™ the FDA-approved humanized monoclonal antibody, and where herstatin, unlike Herceptin™, is a naturally occurring protein having a high degree of specificity, and binding affinity.

5. Nonetheless, I have conducted and supervised two sets of additional data using models, techniques and cell lines that were available in the art at the time of filing of said patent application, to further confirm and illustrate the *in vivo* therapeutic efficacy of herstatin as originally disclosed and enabled by the soft agar cell growth experiments and other teachings therein. The ***first*** data set relates to herstatin's efficacy in inhibiting a variety of additional human cancer cell lines that over-express HER-2 and/or the EGF receptor, and further shows that herstatin's efficacy is as good or better than that of Herceptin™, the clinically approved humanized anti-HER-2 monoclonal antibody. The ***second*** data set relates to herstatin's *in vivo*

stability, and its ***substantial in vivo*** efficacy in inhibiting xenografts of MCF7/HER-2 human breast cancer cells in nude mice.

6. ***First***, I have conducted and supervised a series of concurrent tests using techniques and cell lines that were available in the art at the time of filing, to illustrate that the originally disclosed therapeutic efficacy of herstatin in inhibiting the growth of SKOV-3 and 17-3-1 cells is readily applicable, as disclosed, to a variety of human cancer cell lines that over-express HER-2 and/or the EGF receptor. The data, using purified recombinant herstatin, is summarized in the following Table:

Cell Line	Tissue Source	HER-2	EGFR	Inhibition (% control)
HBL-100	Normal Epithelium	+	+	-7.4*
MCF-7	Breast Carcinoma (Estrogen-dependent)	+	+	-7.3*
BT474	Breast Carcinoma	+++	+	45.9**
SKBR3	Breast Carcinoma	+++	+	51.4***
SKOV3	Ovarian Carcinoma	+++	+	57.9***
NCI-N87	Gastric Carcinoma	++	++	40.9**
DU145	Prostate Carcinoma	+	++	30.0**
A431	Epidermoid Carcinoma	+	+++	36.3***
KB	Head and Neck SS Carcinoma	+	++	51.2***

* ($p>.1$)
** ($p<.05$)
*** ($p<.001$)

For the experiments of the above Table, His-tagged herstatin was expressed in S2 insect cells and purified using nickel affinity chromatography followed by Con A lectin affinity chromatography. Test cells were plated at low density, and quadruplicate wells of cells were treated at day 2 post-plating with various amounts of herstatin, or with control vehicle in low serum. The wells were again treated with herstatin at day 4 post-plating, and live cell assay (MTS) was conducted at day 5.

As previously disclosed in the above-identified patent application, herstatin substantially inhibited human carcinoma cell lines (the cell morphologies indicate cell death). Non-tumorigenic epithelial cells and estrogen responsive breast carcinoma cells (MCF-7) were not

inhibited by herstatin. Herstatin produced in mammalian cells inhibits HER-2 overexpressing carcinoma cells at 1-2 nM.

7. Additionally, I have directly compared the efficacy of herstatin (of the instant invention) with that of Herceptin™ (the clinically approved humanized anti-HER-2 monoclonal antibody) against SKOV3 (ovarian carcinoma), SKBR3 (breast carcinoma) and NCI-N87 (gastric carcinoma), and have found that herstatin is either as effective, or significantly more effective than Herceptin™. The results are shown in **FIGURE 1** (attached hereto) entitled "Direct comparisons of herstatin and Herceptin on breast, ovarian, and gastric carcinoma cells.

8. ***Second***, I have shown that purified recombinant human herstatin (obtain from cultured insect cells) is significantly stable (>1 hour) and nontoxic when injected at up to 20 mg/kg into nude mice (*see* **FIGURE 2**, attached hereto), fully consistent with, and further confirming the teachings of the above-identified patent application.

9. Additionally, I have now demonstrated that purified recombinant herstatin has ***substantial in vivo*** efficacy in inhibiting the growth of xenografts of MCF7/HER-2 human breast cancer cells in nude mice (*see* **FIGURES 3** and **4**, attached hereto). This data was obtained using HER-2 transfected MCF7 (MCF7/HER-2), one of the most commonly used art-recognized models for HER-2 overexpressing breast cancer. In this model, the tumorigenic growth of MCF7 cells is greatly enhanced by overexpression of HER-2 (Kurokawa, H., et al., *Cancer Res.*, 60:5887-94, 2000; Liu, Y., et al., *Breast Cancer Res. Treat.*, 34:97-117, 1995; Pietras, R.J., et al., *Oncogene*, 10:2435-46, 1995, all attached hereto as **EXHIBIT A**). Additioinally, it is known that the tumorigenic growth of MCF7/HER-2 is inhibited by Herceptin™, the clinically approved humanized anti-HER-2 monoclonal antibody marketed by Genentech (Pietras, R.J., et al., *Oncogene*, 10:2435-46, 1995).

Specifically, MCF7/HER-2 cells ($1x10^7$) were implanted into the hind flanks of 4 female athymic (nude) mice. The mice were then injected intraperitoneally with ~30 mg/kg of purified human recombinant herstatin produced in insect cells in 0.1 ml of phosphate buffered saline. The tumors were measured by vernier calipers at the indicated times and the tumor volume was

calculated using the formula: Volume = length/2 x width^2. As shown in **FIGURE 3**, Tumors in three mice initially grew to a volume of ~170 mm^3 and a fourth mouse, #21, grew to a volume of about 450 mm^3. Following the second injection of herstatin, the tumor size in all four mice was reduced and exhibited increased reduction with subsequent injections until the tumor volume was descreased from 5-to 10-fold. As an *internal control* for tumor viability, we examined the effects of withdrawal of herstatin. On day 13 the injections of herstatin were stopped. By day 21, tumors in #23 and #25 exhibited significant growth. To test for possible toxic side effects of Herstatin, the mice were weighed and the indicated time points (**FIGURE 4**). There was no significant effect of herstatin injection on the weight of the mouse, and mouse weight did not correlate with tumor shrinkage or recovery. **These data demonstrate that i.p. administration of purified herstatin caused regression of viable tumors, with no apparent toxic side effects.** In comparison with previously published studies testing Herceptin™ in the MCF7/HER-2 model, while Herceptin™ inhibits the growth of the tumors in a cytostatic manner, herstatin appears to cause ***regression*** of the tumors. This *in vivo* data substantially confirms the clinical utility of Herstatin against human breast cancer cells as disclosed, taught and enabled in the above-identified patent application.

10. In conclusion, techniques and methods available in the art at the time of filing have been used to further confirm the therapeutic utility of herstatin as originally taught, disclosed and enabled in the above-identified pending patent application. Herstatin inhibits HER-2 family receptors from outside the cell, and recombinant bioactive herstatin has equal or better efficacy than Herceptin™, both *in vitro* and *in vivo* against a clinically validated target (HER-2 receptor) and validated models of human cancers. Recombinant herstatin is stable when injected into mice, consistent with its natural occurrence in particular tissues. Herstatin has therapeutic efficacy ***in vivo*** as described, taught and enabled in the above-identified patent application.

11. I further declare that all statements made herein of my own knowledge are true and that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Gail M. Clinton

State of Oregon)
) ss.: Gail M. Clinton
County of Multnomah)

On this 21st day of November, 2002, before me, a Notary Public in and for the State and County aforesaid, personally appeared Gail M. Clinton, to me known and known to me to be the person of that name, who signed and sealed the foregoing instrument, and he acknowledged the same to be her free act and deed.

OFFICIAL SEAL
JACQUELINE M WELLS
NOTARY PUBLIC-OREGON
COMMISSION NO. 361209
MY COMMISSION EXPIRES SEPT 12, 2006

Notary Public

Commission expires Sept. 12, 2006

Direct comparisons of herstatin and Herceptin on breast, ovarian, and gastric carcinoma cells

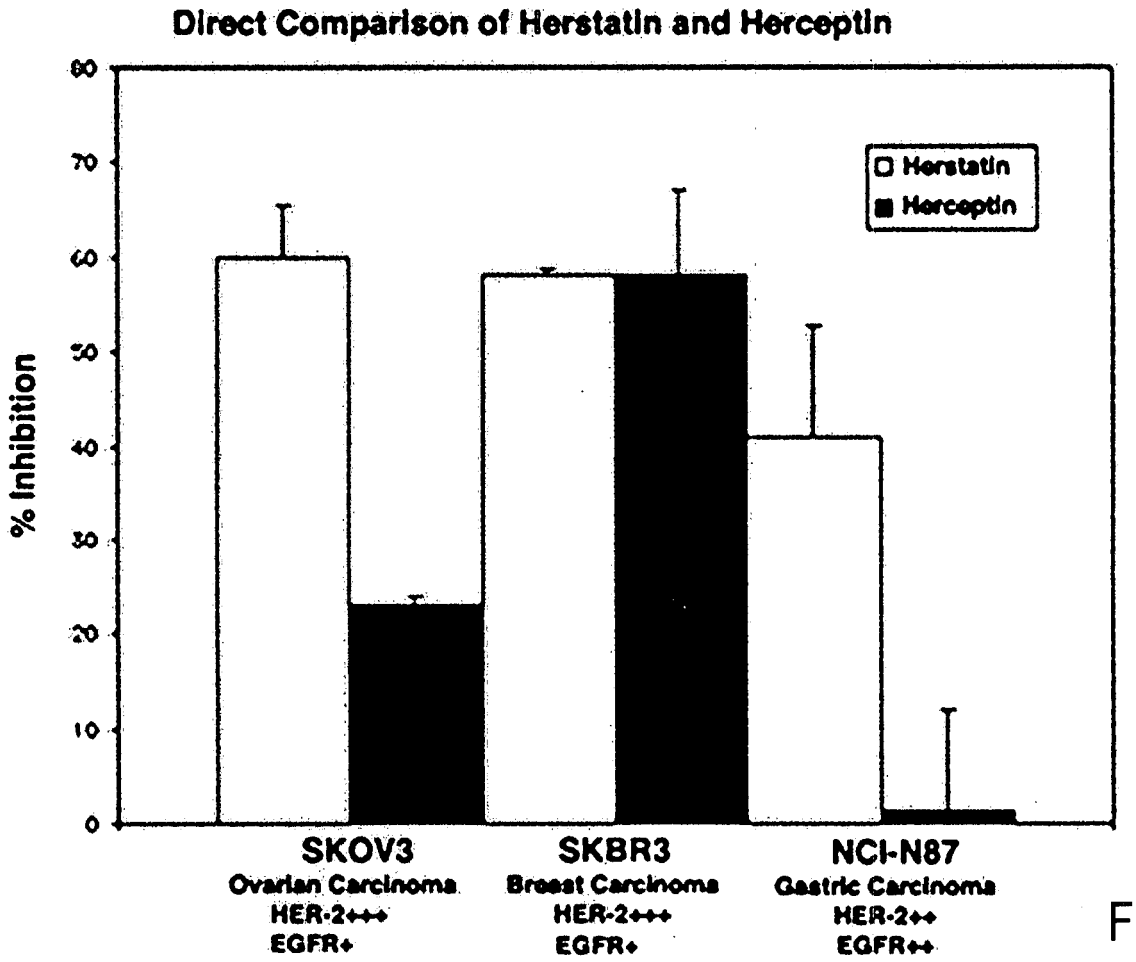


Fig. 1

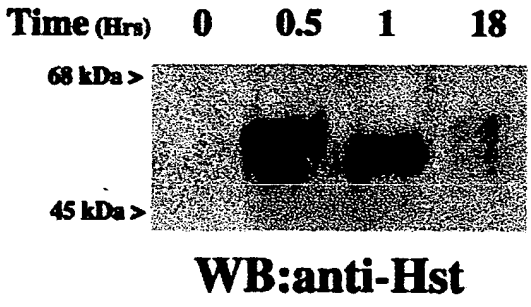


Fig. 2

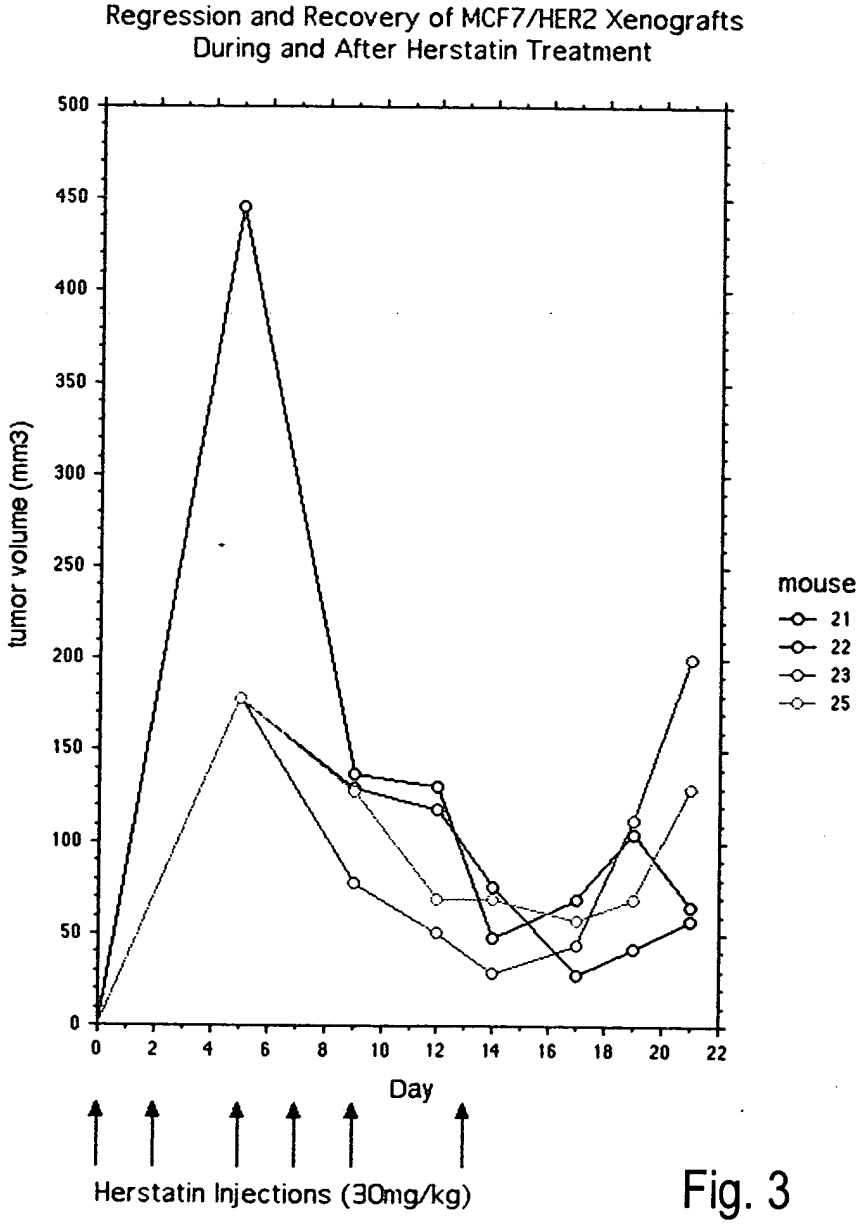
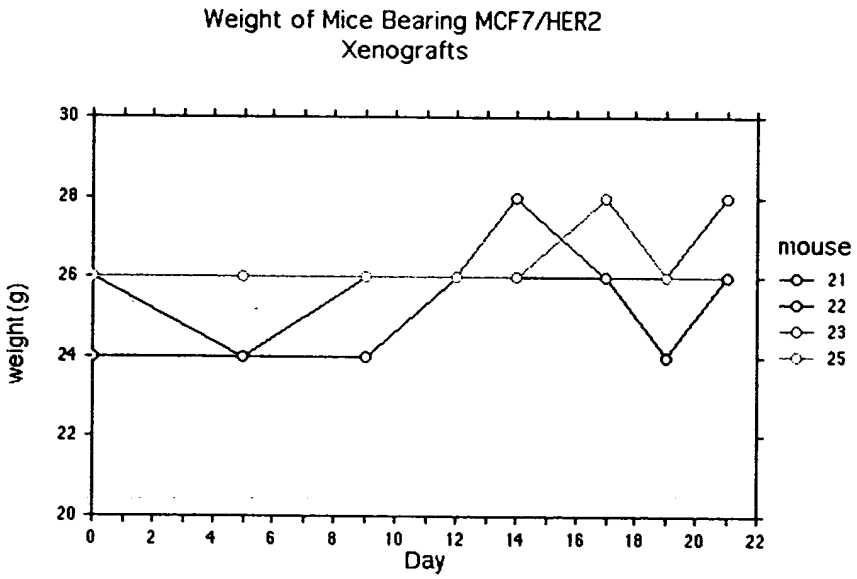

Regression and Recovery of MCF7/HER2 Xenografts
During and After Herstatin Treatment
tumor volume (mm3)
500
450
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18
20
22
Day
mouse
21
22
23
25
Herstatin Injections (30mg/kg)

Fig. 3


Weight of Mice Bearing MCF7/HER2 Xenografts
weight (g)
30
28
26
24
22
20
0
2
4
6
8
10
12
14
16
18
20
22
Day
mouse
21
22
23
25

1.

Fig. 4

Expression and purification of Herstatin. To produce quantities of recombinant Herstatin for testing in animal models, we stably transfected S2 insect cells with a Herstatin expression vector. Herstatin was secreted into the media of stably transfected insect cells. Herstatin was purified from the media by nickel affinity chromatography (NINTA Agarose) and compared side by side with Herstatin produced in bacteria. The insect produced material was about 55 kDa compared to 50 daltons for the bacterial protein. The size difference is due to glycosylation that occurs in the insect cells. The purified material was >90% purity indicated by Coomassie staining (lower panel) and reacted with Herstatin specific antibodies as indicated by Western blot analysis (upper panel). The purified Herstatin was further fractionated by gel filtration. The yield of purified Herstatin is 20-30 mg from 1 L of media conditioned by the cultured insect cells.

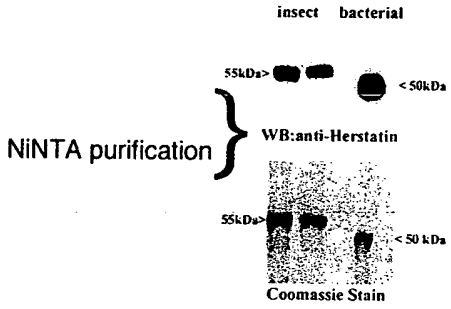


Fig. 5